From Genes to Genomes: Beyond Biodiversity in Spain's Rio Tinto

LINDA A. AMARAL ZETTLER[1], MARK A. MESSERLI[1,2], ABBY D. LAATSCH[1], PETER J. S. SMITH[2], AND MITCHELL L. SOGIN[1,*]

[1] *The Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts; and* [2] *The BioCurrents Center, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts*

Spain's Rio Tinto, or Red River, an example of an extremely acidic (pH 1.7–2.5) environment with a high metal content, teems with prokaryotic and eukaryotic microbial life. Our recent studies based on small-subunit rRNA genes reveal an unexpectedly high eukaryotic phylogenetic diversity in the river when compared to the relatively low prokaryotic diversity. Protists can therefore thrive in and dominate extremely acidic, heavy-metal-laden environments. Further, because we have discovered protistan acidophiles closely related to neutrophiles, we can hypothesize that the transition from neutral to acidic environments occurs rapidly over geological time scales. How have these organisms adapted to such environments? We are currently exploring the alterations in physiological mechanisms that might allow for growth of eukaryotic microbes at acid extremes. To this end, we are isolating phylogenetically diverse protists in order to characterize and compare ion-transporting ATPases from cultured acidophiles with those from neutrophilic counterparts. We predict that special properties of these ion transporters allow protists to survive in the Rio Tinto.

Earth harbors many extreme environments. Previous investigations of the microbial diversity in these environments have been constrained by preconceived notions about the range of habitability for both eukaryotic and prokaryotic microorganisms. We have been exploring the genetic and physiological diversity of organisms living at pH extremes, both acidic (pH < 3) and alkaline (pH > 10). Our research ranges from environments like the warm (45 °C), acidic (pH 2.7) Nymph Creek in Yellowstone National Park to temperate alkaline lakes in the Sandhills region of western Nebraska. The focus of this report is the acidic, heavy-metal-rich Rio Tinto in southwestern Spain.

The Rio Tinto flows 100 km through the world's largest pyritic (FeS_2) belt. The river gets its red color from the high levels of iron dissolved in its acidic waters (pH ~2.0). Ferric hydroxide (Fe^{3+}/$Fe(OH)_3$, pKa 2.5) and SO_4^{2-}/HSO_4^{-} (pKa 2.0) act as buffers to maintain the pH of the river at about 2. The concentration of iron can be as high as 20 g/l, and the river also contains other heavy metals at concentrations orders of magnitude higher than those in typical freshwater environments.

Much of the past research on the Rio Tinto has focused on the prokaryotes that play an important role in shaping the acidic environment of the Rio Tinto through their metabolism of iron-rich pyrite and chalcopyrite. Recent paleontological research shows that iron-oxidizing bacteria existed in the Rio Tinto river basin 300,000 years ago, long before its 5000-year mining history (Leblanc *et al.*, 2000). Other chemolithotrophs such as sulfur-oxidizing bacteria and archaea also contribute to the river's probably ancient ecosystem structure (Gónzalez-Toril *et al.*, 2001).

Some of these prokaryotes, along with fungi, contribute to the formation of biofilms on the surface of rocks. These biofilms, in turn, are the site of metal and mineral precipitation that ultimately forms stromatolites. Biofilms provide a substrate for communities to develop within the river. However, in many parts of this river basin, eukaryotic
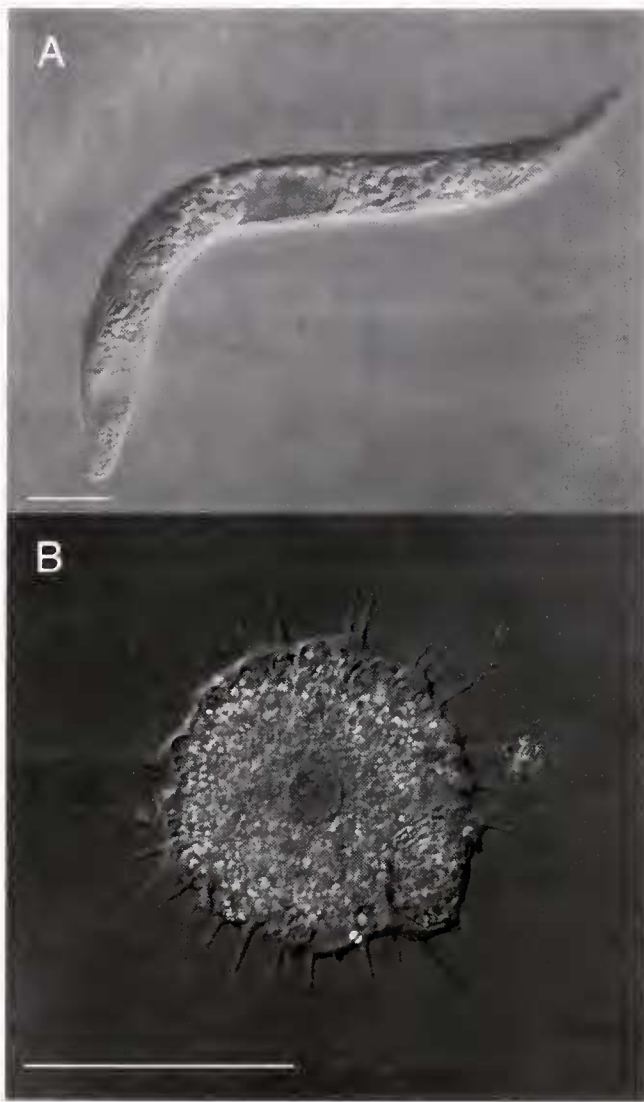
Figure 1.   Some representative eukaryotes found in the Rio Tinto. (A) *Euglena* cf. *mutabilis*; scale bar = 10 μm. Photomicrograph by Linda Amaral Zettler and David Patterson. (B) A heliozoan, most likely belonging to the genus *Actinophrys*; scale bar = 100 μm. Photomicrograph by Linda Amaral Zettler and Erik Zettler.

microbes (Fig. 1) are the major contributors of biomass (López-Archilla *et al.*, 2001). Eukaryotes not only form the foundations of some of these biofilm communities, but they are also conspicuous inhabitants of them (López-Archilla *et*
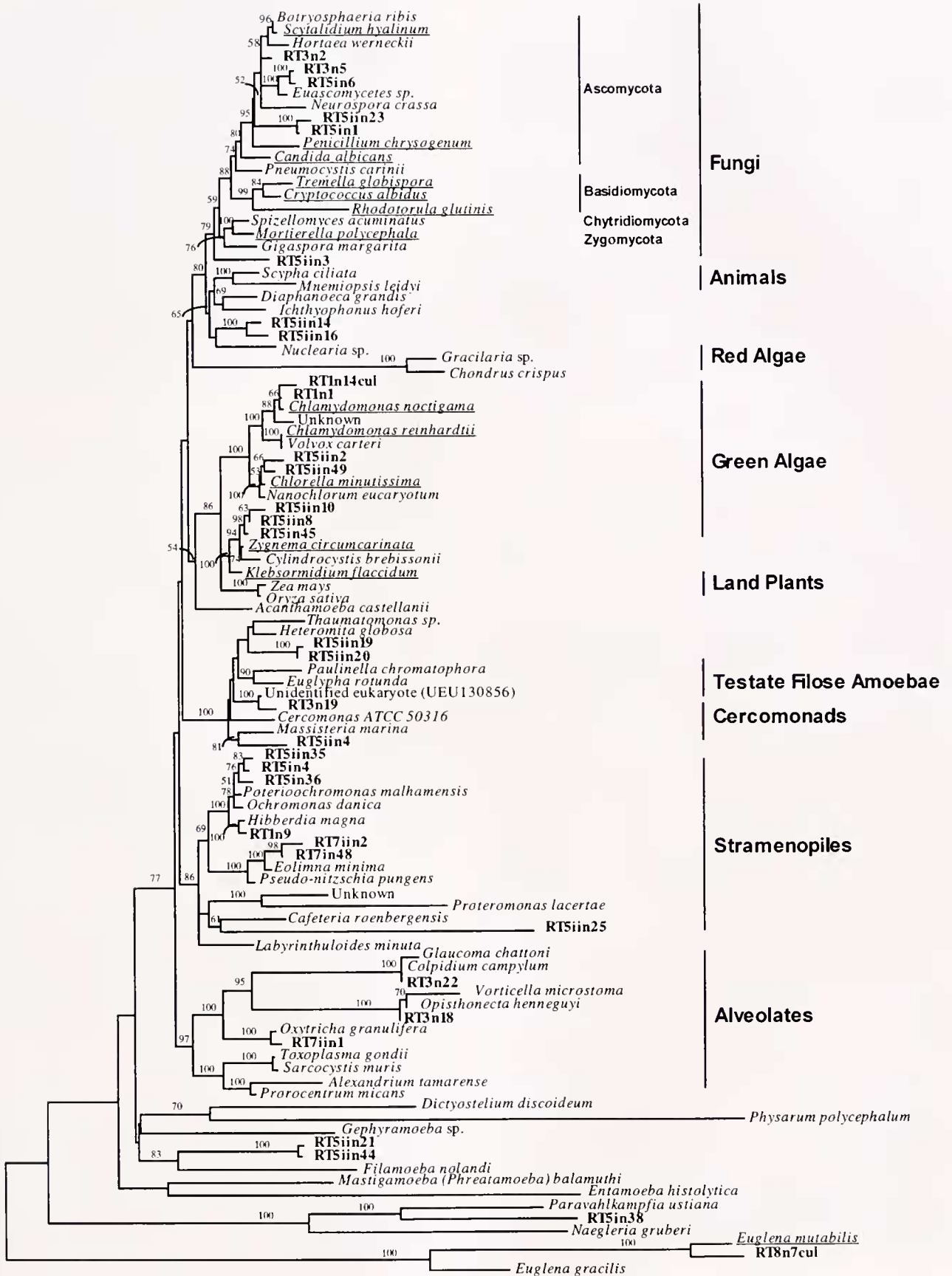
al., 1993, 1994, 1995; López-Archilla and Amils, 1999). This makes the Rio Tinto system unique among acidic environments described to date.

Figure 2 shows a phylogenetic tree depicting the breadth of eukaryotic diversity in the river. In our rRNA gene diversity survey of biofilm samples, we obtained sequences representing most of the major eukaryotic lineages, including the fungi, animals, green algae, land plants, stramenopiles, and alveolates (Amaral Zettler, 2002). Many of the sequences from the Rio Tinto represent photosynthetic lineages that were previously identified employing light microscopy (López-Archilla *et al.*, 2001). These included members of the Euglenozoa (euglenids), Chlorophyta (chlamydomonad and chlorella-like relatives), Viridiplantae (*Zygnema*-relatives), and Bacillariophyta (diatoms). However, our molecular data also unveiled a significant nonphotosynthetic component to the Rio Tinto. Phagotrophic lineages such as the ciliates, cercomonads, and vahlkampfiid amoebae, as well as heterotrophic fungi and possibly myxotrophic lineages—as known to occur in members of the stramenopiles such as *Ochromonas* (Porter *et al.*, 1985)— also populate the Rio Tinto.

We discovered a diversity of fungi that escaped detection using traditional identification methods. None of our fungal clones showed high similarity to those species already described from the Rio Tinto. Fungi undoubtedly play an important role in community structure in the Tinto since most of them are metal resistant and can sequester specific metals (Durán *et al.*, 1999). Such sequestration of potentially toxic metals could allow less tolerant species to exist where they might not have otherwise, possibly enhancing biodiversity in these areas. Similar studies on metal sequestration have not been conducted on protists living in the Tinto. We hope to apply new tools in genome science to address these questions surrounding the rich microbial diversity of the Rio Tinto.

Our studies also revealed a diversity that we were not able to readily characterize using molecular techniques. Clones such as RT5iin16 and RT5iin14 are most likely examples of novel eukaryotic lineages that at best branch at the base of the animal-fungal-nucleariid radiation. Other clones (RT5iin21 and RT5iin44) branched with the recently sequenced filose amoeba *Filamoeba nolandi*, whose own taxonomic placement is equivocal. Because our study was

Figure 2.   A minimum evolution phylogeny for small subunit rRNA genes using a likelihood model. Bold letters indicate environmental clones. "RT" indicates the sequence is from the Rio Tinto, and "cul" indicates cultured species. Underlined taxa represent genera that have been identified in the river based on microscopic observation. Sampling sites were as follows: RT1, La Palma; RT3, Berrocal Upper; RT5i, the Origin, black filamentous biofilm; RT5ii, the Origin, green filamentous biofilm; RT7i, Anabel's Garden green biofilm; RT7ii, Anabel's Garden yellow biofilm. Bootstrap support values are shown, and the scale bar represents the number of substitutions per site. GenBank accession numbers AY082969-AY083001.

96 *Botryosphaeria ribis*
Scytalidium hyalinum
58 *Hortaea werneckii*
RT3n2
100 RT3n5
52 100 RT5in6
Euascomycetes sp.
95 *Neurospora crassa*
100 RT5iin23
RT5in1
80 *Penicillium chrysogenum*
74 *Candida albicans*
88 *Pneumocystis carinii*
84 *Tremella globispora*
59 99 *Cryptococcus albidus*
Rhodotorula glutinis
79 100 *Spizellomyces acuminatus*
Mortierella polycephala
76 *Gigaspora margarita*
RT5iin3
80 100 *Scypha ciliata*
Mnemiopsis leidyi
69 *Diaphanoeca grandis*
Ichthyophonus hoferi
65 100 RT5iin14
RT5iin16
Nuclearia sp.
100 *Gracilaria sp.*
Chondrus crispus
RT1n14cul
66 RT1n1
88 *Chlamydomonas noctigama*
100 Unknown
100 *Chlamydomonas reinhardtii*
Volvox carteri
100 66 RT5iin2
53 RT5iin49
Chlorella minutissima
100 *Nanochlorum eucaryotum*
86 63 RT5iin10
98 RT5iin8
94 RT5in45
Zygnema circumcarinata
54 *Cylindrocystis brebissonii*
100 *Klebsormidium flaccidum*
100 *Zea mays*
Oryza sativa
Acanthamoeba castellanii
Thaumatomonas sp.
Heteromita globosa
100 RT5iin19
RT5iin20
90 *Paulinella chromatophora*
Euglypha rotunda
100 Unidentified eukaryote (UEU130856)
RT3n19
100 *Cercomonas ATCC 50316*
Massisteria marina
81 RT5iin4
83 RT5iin35
76 RT5in4
51 RT5in36
78 *Poterioochromonas malhamensis*
100 *Ochromonas danica*
Hibberdia magna
69 100 RT1n9
98 RT7iin2
100 RT7in48
Eolimna minima
100 *Pseudo-nitzschia pungens*
100 Unknown
Proteromonas lacertae
61 *Cafeteria roenbergensis*
RT5iin25
Labyrinthuloides minuta
Glaucoma chattoni
100 *Colpidium campylum*
95 RT3n22
70 *Vorticella microstoma*
100 *Opisthonecta henneguyi*
100 RT3n18
100 *Oxytricha granulifera*
RT7iin1
100 *Toxoplasma gondii*
100 *Sarcocystis muris*
100 *Alexandrium tamarense*
Prorocentrum micans
70 *Dictyostelium discoideum*
Physarum polycephalum
Gephyramoeba sp.
100 RT5iin21
83 RT5iin44
Filamoeba nolandi
Mastigamoeba (Phreatamoeba) balamuthi
Entamoeba histolytica
100 *Paravahlkampfia ustiana*
100 RT5in38
Naegleria gruberi
Euglena mutabilis
100 RT8n7cul
Euglena gracilis
77
86
97

Fungi

Ascomycota

Basidiomycota
Chytridiomycota
Zygomycota

Animals

Red Algae

Green Algae

Land Plants

Testate Filose Amoebae

Cercomonads

Stramenopiles

Alveolates

0.05 substitutions/site

not exhaustive, we surmise that there are still more undiscovered novel lineages in the river.

Despite our growing knowledge of the Tinto's eukaryotic diversity, we know little about the role eukaryotes play in shaping the varied ecosystems that occur along the river. For example, we do not know if these biofilm communities have microenvironments that enhance survival of their members. Could fungal metal sequestration protect nontolerant species? Furthermore, we know little about how these organisms have evolved adaptations to extreme concentrations of acid and metals.

To explore these questions, we have been isolating organisms from the river for *ex situ* physiological experiments. We have established monocultures of *Chlamydomonas* sp., *Euglena* cf. *mutabilis*, *Chlorella* sp., and *Vannella* sp. isolated from enrichments of river water and are currently exploring the physiology of these protists from extreme environments.

We have initiated our physiological studies on an acidophilic species of a chlamydomonad alga isolated from the river—*Chlamydomonas* sp. Our first question about the physiology of the Tinto acidophiles was the nature of the cytosolic pH (pH$_i$). There are published reports of acidophiles from all domains of life with internal pH values that deviate from neutral—these include the archaebacterium *Picrophilus oshimae*, pH$_i$ = 4.6 (van de Vossenberg *et al.*, 1998); the eubacterium *Bacillus acidocaldarius*, pH$_i$ = 5.6–5.8 (Thomas *et al.*, 1976); and the eukaryotic alga *Euglena mutabilis*, pH$_i$ = 5.0–6.4 (Lane and Burris, 1981). Using the fluorescent H$^+$ indicator BCECF, we determined that our acidophilic chlamydomonad isolate maintains an average internal pH of 6.6 at an external pH of 2 (M. A. Messerli, L. A. Amaral Zettler, S.-K. Jung, P. J. S. Smith, and M. L. Sogin, unpubl.). Our other isolates await similar measurements.

Given that there is a 40,000-fold difference in hydrogen ion activity between the inside and the outside of these cells, we propose the existence of active transport mechanisms that help these organisms regulate their internal pH. We hypothesize that novel diversity in H$^+$-ATPases may explain the ability of different protist species to thrive in the low pH, high-metal Rio Tinto environment. There are two major families of H$^+$-ATPases: the V/F/A-ATPases and the P-type-ATPases. The V-type ATPases can occur in the plasma membrane of eukaryotes (but are more commonly associated with vacuolar membranes) and consist of at least 11 subunits and a molecular mass approaching 10^6 Da. In contrast, eukaryotic P-type ATPases consist of either monosubunits (as with H$^+$-ATPases) or a hetero-subunit (alpha and beta, as found in the Na$^+$/K$^+$-ATPases and H$^+$/K$^+$-ATPases); have a molecular weight of about 100 kDa; and form a phosphorylated intermediate during the course of ATP hydrolysis. Indirect evidence of novel ATPases comes from studies of the protozoan parasite *Leishmania dono-*

vani, which has the ability to switch between living in a neutral environment, pH 7.5, as a promastigote (flagellated stage) and in an acidic environment, pH 5.0, as an amastigote (nonflagellated stage) (Meade *et al.*, 1989). The plasma membrane of this organism contains a P-type ATPase that has two isoforms with slightly different sequences. Isoform 1a is expressed in both promastigotes and amastigotes, whereas isoform 1b is expressed more abundantly in the amastigotes (Meade *et al.*, 1989). This difference suggests the use of a sequence change to accommodate the acidic condition. Modifications to ion regulatory machinery might be reflected by convergent amino acid substitution patterns or by accelerated rates of change in acidophilic protist lineages, as revealed in phylogenetic analyses. For example, portions of membrane-bound V- and P-type ATPases that are exposed to the acidic external environment may display different amino acid substitution patterns than do domains that face the cytoplasm.

We are currently using degenerate primers designed against two conserved regions, the phosphorylation site and the ATP-binding site, to amplify members of the P-type superfamily of ion transporters. Thus far, all of our clones fall into the heavy-metal P-type class but may represent different metal transporters. We have found more diverse sequences in the acidophilic *Chlamydomonas* than in the neutrophilic *C. reinhardtii*. We are screening additional clones for H$^+$-transporting ATPases.

Once we obtain ion-transporter sequence information from these acidophiles, we will focus on correlating the expression of these transporters in space and time to biogeochemical characteristics in the river. This will bring us beyond the study of biodiversity in the river to questions at the heart of potential genomic interactions between members of the microbial consortia. With this kind of approach, we may also be able to determine whether symbiotic interactions are occurring in this environment.

Acknowledgments

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